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***Escherichia coli* pathotypes from Ecuador: association with diarrhea and antibiotic resistance**

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***Escherichia coli* pathotypes from Ecuador: association with diarrhea and antibiotic resistance**

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RESUMEN

Escherichia coli diarreogénica (DEC) es una importante causa de diarrea en el mundo en desarrollo y la detección de estas bacterias y su perfil de resistencia a los antibióticos es necesario para una efectiva terapia. En este estudio, nosotros conducimos un estudio microbiológico en 233 muestras de heces, colectadas durante un estudio de caso-control en un hospital y centro de salud en un barrio de bajos ingresos de Quito, Ecuador desde Abril hasta Septiembre del 2014. Nosotros usamos 8 sets de primers de PCR para detectar los distintos patotipos de DEC. La prevalencia total de DEC fue de 30.5% en los casos y 20.2% en los controles (OR 1.76 IC 95% 0.96-3.20, $p=0.06$), *E.coli* difuso adherente (DAEC) fue el patotipo más frecuentemente aislado en casos y controles (15.3% vs. 6.1% respectivamente) y fue el único patotipo con una asociación estadísticamente significativa con diarrea (OR 2.78, IC 95% 1.11-6.96, $p=0.03$). Para nuestro conocimiento este es el primer estudio que investiga este patotipo en el Ecuador. Adicionalmente, patotipos aislados de casos exhibieron significativos altos porcentajes de resistencia antimicrobial para específicos antibióticos, así como también altos niveles de multidrogo resistencia, que los aislados obtenidos de controles.

Palabras clave: diarrea, virulencia, resistencia antibiótica, patotipos de *E.coli*, multidrogo resistencia.

ABSTRACT

Diarrheagenic *Escherichia coli* (DEC) is an important cause of diarrhea in the developing world and the detection of these bacteria and their antibiotic resistance profiles is necessary for effective therapy. In this study, we conducted a microbiological survey of DEC in 233 stool samples, collected during a case control study in a hospital and health center in a low income neighborhood of Quito, Ecuador from April to September 2014. We used 8 sets of PCR primers to detect distinct DEC pathotypes. The overall prevalence of DEC was 30.5% in cases and 20.2% in controls (OR 1.76 CI 95% 0.96-3.20, $p=0.06$), Diffusely adherent *E.coli* (DAEC) was the most frequently detected pathotype in cases and controls (15.3% vs. 6.1% respectively) and was the only pathotype with a statistically significant association with diarrhea (OR 2.78, CI 95% 1.11-6.96, $p=0.03$). To our knowledge this is the first study investigating this pathotype in Ecuador. Additionally, pathotypes isolated from cases exhibited significantly higher levels of antimicrobial resistance to specific antibiotics, as well as higher levels of multidrug resistance, than isolates obtained from controls.

Key words: diarrhea, virulence, antibiotic resistance, pathotype *E.coli*, multidrug resistance.

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PART I

GENERAL INTRODUCTION

GENERAL INTRODUCTION

The normal human microbiota, also known as commensal microbiota are microorganisms present on body surfaces, exposed to the external environment and have an important role in development of the mucosal immune system (Tlaskalová-Hogenová et al., 2004)(Lederberg, 2001). The human microbiota comprises mostly beneficial, harmless bacteria. Pathogenic bacteria, in the other hand, cause disease; these bacteria have factors which allow colonization invasion and damage of the host (Beceiro, Tomas, & Bou, 2013). Virulence is the ability of a pathogen to cause disease via different factors or mechanisms (Clatworthy, Pierson, & Hung, 2007). The study of genomes is the main instrument for assessing the presence of virulence factors or the combination of factors that determine pathogenicity in bacteria. Also, the levels of expression of virulence associated genes can vary to determination of pathogenic and non-pathogenic bacteria (Pitout, 2012).

Escherichia coli is considered a commensal bacteria of the normal gastrointestinal microbiota in humans and animals (Bonnet et al., 2009), (de Verdier, Nyman, Greko, & Bengtsson, 2012), but some variants cause gastrointestinal disease whereas others cause infections outside gastrointestinal tract (Pitout, 2012). The expression of distinct virulence factors determines the *E.coli* pathotype, as well as; the degree of damage to cellular processes that trigger diseases like cystitis, pyelonephritis, sepsis/meningitis and gastroenteritis (Bonnet et al., 2009). Urinary tract infections (UTIs) in humans produced by extraintestinal pathogenic *Escherichia coli* (ExPEC) are a main cause of morbidity and mortality; these have the specific virulence factors to overcome host defenses and cause infection (Johnson, Kuskowski, Gajewski, Sahm, & Karlowsky, 2004).

In recent years, alarming increases in antibiotic-resistant *E.coli* accompanied by increased virulence have been reported (Johnson et al., 2004), (Pitout, 2012). Antimicrobial resistance is a serious problem that stems from overuse of antibiotics in both medical treatment and for agricultural uses. Pathogenic *E.coli* and commensal *E.coli* have different rates of resistance; resistant commensal *E.coli* are considered a reservoir of antimicrobial resistance genes for dissemination to other bacteria and rarely cause disease (Rosengren, Waldner, & Reid-Smith, 2009). Pathogenic bacteria have increased antimicrobial resistance due to intense exposure to antimicrobial agents and at a genetic level due to the physical linkages between antimicrobial genes and virulence genes (Boerlin et al., 2005)(Zhang et al., 2015).

1.1. Genetic of resistance and virulence

Bacteria and their hosts have co-evolved over millions of years, during which time bacteria have adapted to overcome the host immune system. On the other hand, bacteria have more recently evolved antimicrobial resistance (ability of bacteria to resist the action of an antimicrobial agent) (Beceiro et al., 2013). The virulence in pathogenic bacteria is a process that requires two gene classes: the first genes are related with the survival in host and non-host environments and the second virulence genes are genes rarely detected in non-pathogenic organisms and are unique to the pathogens (Groisman & Ochman, 1996).

Pathogenic bacteria evolved by the acquisition of pieces of foreign genetic material encoding virulence factors such as toxins and adherence factors. These virulence genes are encoded on mobile DNA genetic elements that can be transmitted to other microorganisms such as nonpathogenic bacteria or closely related species. Also, virulence genes can be found as chromosomal inserts or pathogenicity islands, for

example EPEC and EHEC have virulence genes *eaeA* and *espB* found within a 35-kb insert that also is inserted within selenocysteine tRNA gene (Finlay, 1997).

The pathogenicity island responsible for pathogenic behavior in enteropathogenic *E.coli* (LEE locus) has 35 Kb and mediates attaching and effacing lesions on intestinal epithelial cells. This locus LEE is absent in laboratory strains. In the same chromosomal location could be found another pathogenicity island PAI-1 (Groisman & Ochman, 1996).

1.2. Dissemination of genes involved in virulence and resistance

Horizontal gene transfer is the mechanism by which bacteria acquire genes associated with virulence and resistance (Ochman, Lawrence, & Groisman, 2000), (Martinez & Baquero, 2000), (Martínez, 2013). Plasmids and transposons may have led to resistance genes of antibiotic-producing organisms (Pang, Brown, Steingrube, Wallace Jr., & Roberts, 1994); it is thought that pathogenicity islands were acquired by horizontal gene transfer (Groisman & Ochman, 1996), (Finlay, 1997). Next, we described the mechanisms and participants involved in the dissemination.

Mutation and recombination. Gene mutation and recombination are different processes involved in antibiotic resistance and virulence phenotypes (Martínez & Baquero, 2002). The mutation process has been studied in dividing bacteria, where it was observed that mutations occur as the result of errors during the DNA replication process. The intrinsic genes that could be antibiotic resistance mutants are genes required for the entry of antibiotics (target-access mutations), genes required in the protection of the target from the drug (target-protection mutations) (Martinez & Baquero, 2000). In the case of virulence, the mutations in intrinsic genes could help

generate more virulent phenotypes in opportunistic bacteria (Martínez & Baquero, 2002).

Recombination regulates and optimizes the expression of ancient genes producing a low-level resistance phenotype and successful pathogenic clones. Some events of recent evolution of bacteria into an infectious phenotype by recombination have been exposed in recent years; for example the recombination between different sets of genes of *S.pneumoniae* produces rearrangements in the capsular antigens such as mechanism of defense against host immunity (Martínez & Baquero, 2002), starting homologous recombination of *cps* (capsule polysaccharide gene) region in *K.pneumoniae* ST258 strains responsible for the global spread of KPC occurred your molecular diversification; studies suggests that ST258 clade I strains evolved from clade II (Chen, Mathema, Pitout, DeLeo, & Kreiswirth, 2014).

Plasmids. Plasmids are vectors for the propagation of antibiotic resistance and virulence factors. Antibiotic resistance plasmids can be transported individually and in combination with genes encoding bacteriocins, siderophores and cytotoxins. The combination of virulence and antibiotic resistance factors in the same genetic element can produce coselection (Martínez & Baquero, 2002), the major incompatibility group is *IncF* group implicated in carriage of resistance and virulence genes (Beceiro et al., 2013). In conjugative plasmids (important for propagation of antibiotic resistance genes), the expression of *traT* genes is also essential in biofilms development, phagocytosis and manufacture of pheromones (Martínez & Baquero, 2002). For example the *Escherichia coli* (ETEC) strain EC2173 has a plasmid pTC, a 90 Kb self-conjugative virulence plasmid, these encoding the STa and STb heat-stable enterotoxins and tetracycline resistance (*Tn10* transposon) (Fekete et al., 2012).

Transposons. Transposons are also important for the spreading of antibiotic resistance genes and virulence determinants. Transposons can be integrated in transferable plasmids or conjugated and then integrated into bacterial chromosome (Martínez & Baquero, 2002). For example, in *Shigella flexneri* the aerobactin operon is a virulence factor that is part of a transposable element that is a fragment of a pathogenicity island (Vokes, Reeves, Torres, & Payne, 1999). The relationship between antibiotic resistance and virulence in a common transposon is less known, because the transposons have a mosaic structure with vastly recombinogenic regions (Lawrence, Ochman, & Hartl, 1992).

Phages. Virulence factors in phages and bacteriophage associates transduction of antibiotic resistance factors have been described. For example the Shiga toxin-producing *E.coli* (STEC) serotype O104:H4 has virulence features in common with the enteroaggregative *E.coli* (Beutin & Martin, 2012); a pathotype that previously carried the plasmid that encoded TEM-1 and CTX-M-15, the most prevalent secondary beta-lactamases among clinical isolates of Enterobacteriaceae (Brzuszkiewicz et al., 2011). It is presumed that the Shiga toxin was transduced from other enterohemorrhagic *E.coli* strains (Beceiro et al., 2013). However, the presence of the two determinants (virulence and resistance) on the same phage has not yet been reported due to the size requirements for phage DNAs, the addition of genes may generate loss of other (Martínez & Baquero, 2002).

Gene cassettes. The integron family is the most important family of gene cassettes. They capture antibiotic resistance genes in Gram negative and Gram positive bacteria. Integrons may include antibiotic resistance genes and virulence factors such as the VCR cassettes in the chromosome of *Vibrio cholerae* (Martínez & Baquero, 2002).

1.3. Mechanisms implicated in antimicrobial resistance and virulence

The association between virulence and resistance can be of great benefit to the pathogens, in most cases; either by increasing their resistance with decreased virulence and fitness (Beceiro et al., 2013). Next, we will analyze, some examples involving virulence mechanisms related to antibiotic resistance.

The location of the bacteria within the host can modify the susceptibility of bacteria to antibiotics. The main characteristic of virulence of *Legionella pneumophila* is its ability to multiply within and kill alveolar macrophages; this environment provides protection against host's humoral response and antibiotics, which are effective *in vitro* but not *in vivo*. (Barker, Scaife, & Brown, 1995) conducted the first report of growth of *L.pneumophila* in amoeba (*A.polyphaga*, a strain associated with an outbreak in UK), which affected the surface properties of the bacteria by altering proteins, lipopolysaccharides, and fatty acid content; these changes limit the action of antibacterial molecules to cross the cell envelope. Consequently, the intracellular growth of *L.pneumophila* increases levels of resistance to antimicrobial agents.

The pathogenicity of the bacteria may contribute to its resistance. Methicillin resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial infections worldwide (Herold et al., 2007). Resistance to methicillin and oxacillin is conferred via acquisition of the staphylococcal chromosomal cassette *mec* (SCC*mec*) and due to the emergence of community-associated MRSA (CA-MRSA) to cause infections outside healthcare settings; (Rudkin et al., 2012) were able to explain why the HA-MRSA (healthcare-associated MRSA) is restricted to healthcare environments while CA-MRSA is not. Their main finding was: the expression of the *mecA* gene reduces the ability of HA-MRSA to

secrete cytolytic toxins. In a murine model of sepsis, these authors showed that resistance to methicillin induces changes in the cell wall that affects the bacteria's *agr* quorum sensing system, reduces expression of toxins and therefore decreases the virulence. The emergence by CA-MRSA strains was explained by the decreased expression of penicillin-binding protein 2a (encoded by *mecA*) and maintaining its virulence. In this circumstance, the mechanism of pathogenicity of the bacteria may serve as mechanism for antibiotic resistance, where the acquisition of resistance to oxacillin is associated with the reduction in virulence.

On the other hand, (Queck et al., 2009) identified and characterized phenol-soluble modulins (PSMs) α -type, peptides that represent toxins that contribute to the neutrophil lysis in CA-MRSA. The cytolytic PSM α peptides are encoded in the core-genome located *psmA* operon. These authors identified the new *psm-mec* gene encoded within a *SCCmec* MGE (mobile genetic element) that involves a molecular connection between virulence and antibiotic resistance. This finding suggests that antibiotic resistance and virulence factors can be linked in staphylococcal MGEs. Also Zhang et al., 2015 in their study with pathogenic and commensal *Escherichia coli* isolates in a community with its profile of resistance to 12 antibiotics and presence and absence of known virulence factor genes determined that the co-occurrence of resistance and virulence is due to the pressure of antibiotic selection and genetic characteristics of isolates (Zhang et al., 2015).

Antibiotic resistance factors also favor the bacterial virulence: efflux system. The ability of bacteria to cause disease also depends on their capacity to resist antibiotics, antimicrobial compounds of host as bile acids, fatty acids and components of the immune system such as antimicrobial peptide (Beceiro et al., 2013). The AcrAB-TolC

system is a participant of the resistance-nodulation-division (RND) family. This efflux system confers innate resistance to toxic substances, including antibiotics, dyes, disinfectants and detergents and substances made by the host (such as bile, hormones and host defense molecules). (Buckley et al., 2006), previously demonstrated that AcrB and TolC are required for *S. enterica* serovar Typhimurium SL1344 to colonize in poultry. Webber et al., 2009 generated mutants lacking *acrA*, *acrB* and *tolC* in which they observed a different expression of major operons and proteins involved in pathogenesis. In mutants, the lack of AcrB or TolC caused suppression of chemotaxis and motility genes, while to mutants lacking *acrA* or *acrB* gene, the *nap* and *nir* operons were repressed and mutants grew poorly. Thus, the consequence of the attenuation of *Salmonella* Typhimurium by creating mutant lacking AcrB or TolC decreased the expression of genes implicated in pathogenic process, particularly SPI-1 (*Salmonella* Pathogenicity Island). Therefore, the RND efflux pumps (antibiotic resistance) and the AcrAB-TolC system (virulence) are essential to the biology of *Salmonella* (Webber et al., 2009).

Persister cells. Persisters are microbial populations that are tolerant to antimicrobials (dormant variants that are really not resistant). Also, persisters cells are frequently found in biofilm, described in species such as *P. aeruginosa*, *Candida albicans*, *S. aureus* and *E. coli* (Mulcahy, Burns, Lory, & Lewis, 2010), (LaFleur, Qi, & Lewis, 2010), (Lechner, Lewis, & Bertram, 2012). So far, it is known that in *E. coli*: the SOS stress response activates persister formation, for example fluoroquinolone antibiotics induce the SOS response, turning the expression of the TisB toxin and causing dormancy by decreasing the proton motive force and ATP levels (Lewis, 2010). Therefore, the persistent populations have evolved to adapt, survive and persist in the environment

and this tolerance to antimicrobials is closely linked to the expression of different virulence factors (Beceiro et al., 2013).

Alarmones Guanosine Tetraphosphate. The molecule alarmones guanosine 3', 5'-bis-(diphosphate) (ppGpp) has intracellular signaling, levels are correlated with the expression of virulence characteristics such as survival of stress in *Campylobacter jejuni*, biofilm formation in *E.coli* and *S.mutans*, antibiotic resistance in *E.coli* (Greenway & England, 1999) and *Brucella abortus* and infection persistence in *M.tuberculosis* (Beceiro et al., 2013). Bacteria such as *E.coli*, when experiencing nutrient limitation, decrease their growth and make adjustments in metabolism, the response mediated by the accumulation of 5'-diphosphate 3'-diphosphate (ppGpp), which in turn is the product of the *relA* gene (Greenway & England, 1999), (Pomares, Vincent, Farías, & Salomón, 2008). Also, in *E.coli* the relationship between (p)ppGpp levels and antibiotic resistance has been observed (Wu, Long, & Xie, 2010), where the increased levels of (p)ppGpp intensifies the β -lactam tolerance and mutants lacking RelA are more susceptible to β -lactams (Pomares et al., 2008). (p)ppGpp has been linked with growth, stress, starvation and survival that affect pathogenicity. Consequently; when (p)ppGpp is absent the pathogenicity is compromised. In *S. typhimurium* the accumulation of (p)ppGpp in stationary phase induce *hilA* (master regulator of pathogenicity island 1 (SPI 1) and SPI 2 virulence genes), while that in enterohemorrhagic *E.coli* (EHEC), its adherence capacity, expression in the enterocyte effacement pathogenicity island locus depends of the expression of *relA*, *spoT* and *dskA* genes (Potrykus & Cashel, 2008).

1.4. The cross roads of virulence and antibiotic resistance

Virulence and epidemicity (capacity to produce epidemics) are essential to cause disease, a phenomenon that does not produce non-virulent bacteria. Many pathogenic bacteria are under antibiotic pressure because they cause infection, therefore; pathogens can be not merely virulent and epidemic but also antibiotic resistant (Martínez & Baquero, 2002); because in a disease the pathogens are present and antibiotic therapy is administered, but in absence of infection the probability of development resistance is lower (Beceiro et al., 2013).

Resistance may have a “direct” cost in bacterial fitness and an “indirect” cost due to the presence of antibiotic resistance factors in mobile elements such as plasmids, transposons and integrons. Antibiotic resistance may have a fitness cost which may diminish bacterial virulence (Gillespie & McHugh, 1997). An increase in virulence in an antibiotic resistant bacterium is unlikely because susceptible revertants may take over in the absence of antibiotic (Martínez & Baquero, 2002).

The successful spread of resistant bacteria clones has increased resistance levels globally (Martínez & Baquero, 2002). The emergence of resistance occurs mainly in hospitals where antibiotic use is common (Levy & Marshall, 2004). Nosocomial transmission of bacteria many times is the result of lack of sterility or hygiene; non-sterile devices or procedures, hospital food, hands of staff (Palmer, 1980) (Livermore, 2003). Therefore, the spread of resistant clones is successful when the two major components are combined: the antibiotic and genetic resistance factor in an environment or host (Levy & Marshall, 2004).

The superbugs, microbes with enhanced morbidity, mortality and high levels of resistance to the antibiotic classes for their treatment are super resistant strains have

acquired increased virulence and enhanced transmissibility (Davies, J, & Davies, D., 2010), for example *Mycobacterium tuberculosis* strains resistant to four or more of the front-line treatment. Other superbug is the Gram-positive organism methicillin-resistant *S.aureus* (MRSA), a multidrug-resistant strain with enhanced virulence and transmissibility. Community acquired-MRSA has different *mec* gene clusters which include new pathogenicity genes, as the gene encoding the cytotoxic Panton-Valentine leukocidin. Consequently, the use of antibiotics can select for more virulent strains (Wilkinson, 1999).

On the other hand, pathogens such as *N.meningitidis*, *Bordetella pertussis*, *Brucella melitensis*, *Salmonella enterica* serovar Typhi are infrequently resistant to antibiotics (Martínez & Baquero, 2002), by factors such as: i) the cost in the fitness of the bacteria by antibiotic resistance should be tolerable (Andersson & Hughes, 1996), ii) highly virulent organisms have evolved in environments protected from the action of natural antibiotics and less open to competition, iii) the number of commensal bacteria is greater than pathogenic bacteria, therefore; the ability to generate resistance is low (e.g., capacity to develop mutational resistance to penicillins in *S.pyogenes* versus viridans group streptococci) (Baquero, 1997) and iv) the particular niche of a pathogenic bacteria can limit your exposure antibiotic agents and selective pressure for the same.

Finally, the relationship between virulence and resistance in a pathogen depends on four factors: First is the bacterial species, because many microorganisms can evolve in reaction to antibiotic pressure. For example *P.aeruginosa* evolve in response to antibiotic pressure and other microorganisms as *S.pyogenes* continue fully susceptible for treatment of choice, such as penicillin (Beceiro et al., 2013). The second factors

specific virulence and resistance mechanisms. There are mechanisms that are implicated in the two processes (virulence and resistance), such as AcrAB-TolC efflux pump of *E.coli* that exports fatty acids, bile salts and antibiotics; the inactivation of *acrAB* leads to reduced ability to colonize the intestinal tract (Zgurskaya & Nikaido, 1999), PhoP regulates resistance to colistin in *P.aeruginosa*, the change in LPS produces decrease in virulence by low production of biofilm (Gooderham et al., 2009). The third factor is the environment or ecological niche, determined by the development of infection and present stimuli: the presence or absence of some factors (e.g., depletion of iron), the antibiotic concentration and NaCl concentration (e.g., in *A.baumannii* augments the resistance to several antibiotics by upregulation of efflux pumps) (Beceiro et al., 2013) (Hood, Jacobs, Sayood, Dunman, & Skaar, 2010). The fourth factor is the host (immune system); because the coselection process occurs within the host. The nosocomial environment, resistant and/or virulent bacteria are selected by antibiotic pressure, but in environments with small concentrations of antibiotics (community environment) common mechanism of resistance and virulence can be selected against (Beceiro et al., 2013).

Therefore, a Darwinian model would be present in the relationship between resistance and virulence, where the traits that confer a benefit will be selected and fixed with a positive effect increased resistance plus augmented virulence or a negative effect, augmented resistance with reduced virulence. However, it may also occur, that increased virulence leading to decrease of resistance (Beceiro et al., 2013).

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PART II

SCIENTIFIC PAPER

***Escherichia coli* pathotypes from Ecuador: association to diarrhea and antibiotic resistance**

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INTRODUCTION

Diarrhea is the second leading cause of death in children under five years and each year produces around 760,000 deaths (1). Among microorganisms causing diarrhea are viruses, bacteria and parasites (2). Pathogenic *Escherichia coli* is one of the primary bacteria that causes diarrheal disease in developing countries (3). Six main pathotypes of diarrheagenic *E.coli* have been associated with disease on the basis of epidemiological and clinical features and specific virulence determinants: enteropathogenic *E.coli* (EPEC), enterohemorrhagic *E.coli* (EHEC), enterotoxigenic *E.coli* (ETEC), enteroaggregative *E.coli* (EAEC), enteroinvasive *E.coli* (EIEC) and diffusely adherent *Escherichia coli* (DAEC) (4).

Antimicrobial resistance is more common among pathogens than among commensal bacteria due to the more intense and repeated exposure of pathogens to antimicrobial agents (5). Clonal expansion and horizontal gene transfer associated with mobile genetic elements such as plasmids, phages and transposons are the most important mechanisms that contribute to the dispersal of antibiotic resistance (6). One intriguing observation is antibiotic resistance genes are linked to virulence genes in pathogens (7). The evolutionary mechanisms that lead to this kind of association could contribute to the emergence of super-pathogens which is a growing problem in nosocomial infections caused by multidrug resistant Enterobacteriaceae. The dispersion of successful clones that combine multidrug resistance and virulence may be fueled by excessive use of antibiotic in medicine and agriculture and increased human movement (8). More studies are needed to understand all the aspects related to the genetic connection between antibiotic resistance and virulence (9)(10).

We investigated the prevalence, association with diarrhea symptoms, and patterns of antibiotic resistance of six pathotypes of *E.coli* isolated from stool from subjects in a low-income urban community of Ecuador.

MATERIALS AND METHODS

Study population. Samples (diarrheal cases and controls) were collected from Enrique Garces hospital and a Chimbacalle local health center in a low income neighborhood in Quito. We initially started recruiting subjects the hospital site, but because recruitment of children at that site proved difficult, we transferred our study to the local health center, in order to obtain samples from the population most at risk of diarrheal illness.

Study design. We conducted a case-control study from April to September 2014. Cases were defined as individuals with three or more loose stools in 24 hours. Controls were individuals who came to the health center or hospital for another reason, and did not have diarrheal symptoms during the past seven days. Subjects were excluded if they reported having taken antibiotics anytime in the prior week, or if they had not lived in Quito for at least six months. Individuals of all ages were eligible to participate in the study, and cases were age-matched with controls using the following age categories: 0-24 months: ± 6 months; 25-60 months: ± 12 months; 61-180 months: ± 24 months; >180 months: any age above 180 months. Surveys were carried out using electronic Android devices, using Open Data Kit program (<http://opendatakit.org>). All the protocols were approved by the Ethics Committees of Emory University and Universidad San Francisco de Quito.

Bacterial culture. Cary-Blair transport media was inoculated with each of the fecal samples using a swab and maintained at 4°C for 48 hours; the rest of the fecal sample

was preserved in nitrogen in cryoconservation tubes. Swabs were cultured on MacConkey's agar media (MKL) for 24 hours, and from these plates 5 lactose positive isolates were randomly selected and non-lactose-fermenting colonies were cultured in Chromocult agar media (Merck, Darmstadt, Germany) (CC) to test for β -glucuronidase activity. Non-lactose-fermenting colonies were identified by biochemical tests as *Shigellae* or *E.coli* using API 20E (BioMérieux, Marcy l'Etoile, France). The 5 isolates were also transferred and cultured in nutrient agar and colonies from the 5 isolates (1 colony per isolate) were pooled together in a tube containing 300 μ l of sterile distilled water and boiled for 10 min to release the DNA. The resulting supernatant was used for PCR testing.

Diarrheagenic *E.coli* identification. Identification of *E.coli* pathotypes was performed with primers designed to detect the presence of specific virulence genes (*bfp*, *lt*, *sta*, *ipaH*, *aggR*, *afa* and *eaeA* genes) in lactose fermenting colonies with modifications to the protocols described in Table 1. Pools positive for *eaeA* gen were tested for *stx1* and *stx2* genes and lactose negative *E.coli* was also tested for the presence of *ipaH* and *afa* gene. For each pool that tested positive for one of the 8 virulence factors, each isolate was retested individually, to identify the isolate responsible for the positive result.

Antibiotic susceptibility testing. All isolates identified as *E. coli* pathotypes were analyzed for their antimicrobial susceptibility by disk diffusion according to the Clinical Laboratory Standards Institute (CLSI) guidelines (clsi.org) on Mueller-Hinton agar. The antibiotics analyzed were: ampicillin(AM,10ug disk), amoxicillin-clavulanic acid (AmC, 20/10mcg disk), cefotaxime(CTX, 30ug disk), ciprofloxacin (CIP, 5ug disk), sulfamethoxazole-trimethoprim (SXT, 1.25/23.75ug disk), gentamicin(CN, 10ug disk), tetracycline (Te, 30 ug disk), imipenem (IPM,10ug disk), chloramphenicol (C, 30ug

disk), sulfisoxazole(G, 250ug disk), streptomycin (S, 10ug disk) and cephalothin (CF, 30ug disk).

Statistical analysis. Chi-square tests and Fisher's exact test were used for group comparisons and multivariate logistic regression was used to calculate odds ratios, adjusting for confounding variables (age and home water treatment). All data were analyzed with Stata 14.0 (StataCorp. LP, College Station, TX).

RESULTS

We collected 118 stool samples from diarrhea cases and 115 from control samples. No statistical differences were observed between cases and controls for age, sex, access to sanitation, recent contact with animals, or travel within the last year. However, cases were significantly more likely to report having treated their drinking water at home (Table 2).

A total of 233 *E.coli* strains were evaluated by PCR. Sixty *E.coli* strains were classified as diarrheagenic *Escherichia coli* (DEC), 36 from cases (17 children and 19 adults) and 24 from controls (19 children and 5 adults). *Shigella* spp. was found in the diarrhea group but no STEC and EHEC strains were isolated.

The prevalence of *E.coli* pathotypes was higher in cases (30.5%) than in controls (20.2%) ($p=0.06$). In cases the most prevalent pathotype was DAEC, followed by ETEC, while in the controls EPEC was the most prevalent pathotype, followed by DAEC. Regarding EPEC, only one isolate (from a case) was typical EPEC (*bfp*+), the rest of the isolates obtained in both cases and controls were atypical EPEC (*eaeA*+, *bfp*-, *stx1*-, *stx2*- genes) (Table 3b). The only pathotype that was associated with diarrhea case status was DAEC (OR 2.78, CI 95% 1.11-6.96, $p=0.03$).

Antibiotic susceptibility results indicated high rates of resistance of pathotypes to ampicillin (62%), sulfamethoxazole-trimethoprim (68%) and sulfisoxazole (70%). All strains were fully susceptible to imipenem, and only 1 isolate (ETEC) was resistant to ciprofloxacin. There were no statistically significant associations between any specific pathotype with any specific antibiotic resistance (Table 4b).

For all antibiotics, a higher frequency of resistance was found in cases versus controls and this difference was higher in magnitude and statistically significant for ampicillin (OR 3.07, CI 95% 1.04-9.09), sulfamethoxazole-trimethoprim (OR 4.14, CI 95% 1.31-13.08) and sulfisoxazole (OR 3.50, CI 95% 1.10-11.09) (Table 5b). The rate of resistance to any antibiotic was also significantly higher in cases versus controls (OR 4.80, CI 95% 1.27-18.11), as was multidrug resistance, defined as resistance to three or more antibiotics (OR 3.51, CI 95% 1.10-11.08).

There were 16 different antibiotic resistance patterns among the diarrheagenic *E.coli* (diarrhea and control strains grouped together). The most common resistance patterns were AM-SXT-G (66.7%), followed by SXT-Te-G (44.4%) and the pattern AM-SXT-Te-G (36.1%). One isolate from a diarrhea case, showed resistance to 9 antibiotics.

For multidrug resistance, results of the multivariate logistic regression, was used adjusting for age and home water treatment, suggest that *E.coli* pathotypes isolated from cases have 7.32 higher odds of being multidrug resistant compared to *E.coli* pathotypes isolated from controls.

DISCUSSION

Our report shows high prevalence of DAEC (15.3% in cases vs. 6.1% in control) and association of this pathotype with diarrhea in low income communities of Ecuador (OR

2.78, CI 95% 1.11-6.96, $p=0.03$). We did not find significant associations between diarrhea and other pathotypes (ETEC, EPEC, EIEC, EAEC and Shigellae), although this could have been a result of an insufficient sample size. EIEC and Shigellae were only found in cases. EPEC and EAEC were higher in controls compared to cases (Table 3b). These trends held when considering the more limited sample set associated when we excluded the hospital samples from the analysis, although this subset analysis limited the power of our results.

In other similar communities in Ecuador, previous reports found EIEC, ETEC, EPEC associated with diarrhea (11)(12)(13)(14), but these studies did not test for EAEC. Factors (such as virulence) related to the pathogen or host factors (such as immunity, gut microbiome, nutritional status) may determine whether an infection causes diarrhea or not (15).

In this study, we also found that antibiotic resistance was more associated with *E.coli* pathotypes causing diarrhea than in those from controls, especially to ampicillin, sulfamethoxazole-trimethoprim and sulfisoxazole. *E.coli* pathotypes from cases were also 3.5 times more likely to have multidrug resistance compared with pathotypes from controls and this association doubled when controlling for age and household water treatment. Our results are in agreement with studies in Peru and Mexico (16)(17), which also found high rates of resistance to ampicillin and sulfamethoxazole-trimethoprim in *E.coli*. Also, these results are similar to those of previous reports carried out in *E.coli* from urinary tract in Ecuador (18). In Ecuador, this *E.coli* resistance profile (trimethoprim-sulfamethoxazole and ampicillin) was similar to that found previously in *E.coli* from urinary infections (19); and was the most frequently found in infectious *E.coli* from fecal samples in rural communities of Ecuador (20)(21). A recent

report indicates high resistance to these antibiotics: ampicillin (78%), trimethoprim-sulfamethoxazole (63%) and cefepime (63%) in *E.coli* from urinary infections in Ecuador (22). We did not find any significant difference in antibiotic resistance among pathotypes nor did we find differences in isolates from different ages or from people treating water.

Our results are in alignment with previous findings which showed that resistance to multiple antibiotics was significantly higher in pathogenic *E.coli* compared to commensal *E.coli* (23)(24)(9)(25), however we showed that pathotypes associated with cases of diarrhea had higher rates of resistance than pathotypes obtained from healthy individuals. These data may indicate that pathotypes with higher resistance may belong to different genetic lineages associated with more virulence(7). This variation in virulence may also explain why we were unable to link some pathotypes with diarrhea in the present study while this association was observed previously in similar communities in Ecuador. Also, despite exclude people who took antibiotics to participate in the study may have included participants not reporting abuse.

In summary, our results corroborate previous studies which suggest that virulence may be linked to antibiotic resistance. Based on our results we postulate that the different lineages of *E.coli* belonging to the same pathotype but which have difference in virulence; the most virulent lineages will be more likely to cause disease and patients carrying these bacteria will be more likely to receive antibiotics which may provide the selective pressure to accumulate antibiotic resistance genes (26).

Finally, to our knowledge EAEC and DAEC were investigated for the first time in Ecuador and DAEC was the pathotype most frequently isolated in this community and associated with diarrhea.

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PART III

TABLES AND FIGURES

Table 1. Primers for conventional PCR for diarrheagenic *E.coli* genes

<i>E.coli</i>	Gene	Primer sequence 5 – 3'	Size (bp)	Reference
EAEC	<i>aggR</i>	5'GTATACACAAAAGAAGGAAGC3'	254	(27)
		5'ACAGAATCGTCAGCATCAGC3'		
ETEC	<i>lt</i>	5'GCGACAAATTATACCGTGCT3'	708	(28)
		5'CCGAATTCTGTTATATATGT3'		
	<i>sta</i>	5'CTGTATTGTCTTTTTCACCT3'	182	(28)
		5'GCACCCGGTACAAGCAGGAT3'		
EPEC	<i>bfp</i>	5'CAATGGTGCTTGCGCTTGCT3'	324	(28)
		5'GCCGCTTTATCCAACCTGGT3'		
	<i>eaeA</i>	5'GACCCGGCACAAGCATAAGC3'	384	(29)
		5'CCACCTGCAGCAACAAGAGG3'		
EIEC	<i>ipaH</i>	5'GCTGGAAAACTCAGTGCCT3'	424	(28)
		5'CCAGTCCGTAAATTCATTCT3'		
DAEC	<i>afa</i>	5'GCTGGGCAGCAAACCTGATACTCTC3'	750	(30)
		5'CATCAAGCTCTTTGTTCGTCCGCCG3'		
STEC	<i>stx1</i>	5'ATAAATCGCCATTCGTTGACTAC3'	180	(29)
		5'AGAACGCCCACTGAGATCATC3'		
	<i>stx2</i>	5'GGCACTGTCTGAACTGCTCC3'	255	(29)
		5'TCGCCAGTTATCTGACATTCTG3'		

Table 2

Demographic data of patients included in the study						
Parameter	Health center			Health center+hospital		
	Case(N=96)	Control(N=102)	p-value	Case(N=118)	Control(N=115)	p-value
Age(year)						
Mean(SD)	16.9(21)	15.7(19.9)	0.60	18.9(21.3)	18.6(21.7)	0.32
Age categories						
<1year	29(30.2%)	29(28.4%)		31(26.3%)	30(26.1%)	
1-15year	31(32.3%)	38(37.3%)		35(30%)	39(33.9%)	
16-30year	14(14.6%)	15(14.7%)		21(17.8%)	16(13.9%)	
>30year	22(22.9%)	20(19.6%)	0.88	31(26.3%)	30(26.1%)	0.83
Sex						
Male	51(53.1%)	46(45.1%)		55(46.6%)	51(44.4%)	
Female	45(46.9%)	56(54.9%)	0.26	63(53.4%)	64(55.7%)	0.73
Sanitation						
Flush toilet	74(77.1%)	78(76.5%)		94(80%)	90(78.3%)	
Diaper	18(18.8%)	16(15.7%)		20(17%)	17(14.8%)	
Latrine*	3(3.1%)	7(6.9%)		3(2.5%)	7(6.1%)	
Open field	0(0%)	1(0.98%)		0(0%)	1(0.9%)	
Cesspool	1(1.0%)	0(0%)	0.46	1(0.85%)	0(0%)	0.42
Reported home water treatment						
No	45(46.9%)	29(28.4%)		54(45.8%)	33(28.7%)	
Si	51(53.1%)	73(71.6%)	0.007	64(54.2%)	82(71.3%)	0.007
Reported recent contact with animals						

No	47(49%)	44(43.1%)		58(49.2%)	50(43.5%)	
Si	49(51%)	58(56.9%)	0.41	60(50.9%)	65(56.5%)	0.39
Reported travel in the last year						
No	47(49%)	42(41.2%)		62(52.5%)	50(43.5%)	
Si	49(51%)	60(58.8%)	0.27	56(47.5%)	65(56.5%)	0.17

*Latrine (space for defecation, without the connection to a sewage system and subsequent treatment)

**Chi square test was used to the comparison between cases and controls ($p \leq 0.05$)

Table 3a

Prevalence of diarrheagenic <i>E.coli</i> and Odds ratio in health center						
	Cases (N=96)	Controls (N=102)	OR(95%CI)	p- value	Cases(n=25 ,%)	Control (n=22),%
All pathotypes	26.0%	21.6%	1.35(0.70- 2.63)	0.36		
Enterotoxigenic <i>Escherichia coli</i>	1.0%	3.9%	0.26(0.03- 2.35)	0.23	1(4%)	4(18.9%)
Diffusely adherent <i>Escherichia coli</i>	15.6%	6.9%	2.51(0.98- 6.47)	0.06	15(60%)	7(31.8%)
Enterobacterial <i>Escherichia coli</i>	1.0%	3.9%	0.26(0.03- 2.35)	0.23	1(4%)	4(18.2%)
Enteroinvasive <i>Escherichia coli</i>	4.2%	0.0%	NA	NA	4(16%)	0(0%)
Enteropathogenic <i>Escherichia coli</i>*	4.2%	6.9%	0.69(0.19- 2.55)	0.58	4(16%)	7(31.8%)
Shigellae	0.0%	0.0%	NA	NA	0(0%)	0(0%)

* A single isolation was typical EPEC (*bfp+* gene)

Table 3b

Prevalence diarrheagenic <i>E.coli</i> and Odds ratio in health center+hospital						
	Cases (N=118)	Controls (N=115)	OR(95%CI)	p- value	Cases(n=36 ,%)	Control (n=24),%
All pathotypes	30.5%	20.2%	1.76(0.96- 3.20)	0.06		
Enterotoxigenic <i>Escherichia coli</i>	5.1%	3.5%	1.49(0.41- 5.41)	0.55	6(16.7%)	4(16.7%)
Diffusely adherent <i>Escherichia coli</i>	15.3%	6.1%	2.78(1.11- 6.93)	0.03	18(50%)	7(29.2%)
Enterobacterial <i>Escherichia coli</i>	0.8%	3.5%	0.24(0.03- 2.15)	0.20	1(2.8%)	4(16.7%)
Enteroinvasive <i>Escherichia coli</i>	3.4%	0.0%	NA	NA	4(11.1%)	0(0%)
Enteropathogenic <i>Escherichia coli</i>*	3.4%	7.6%	0.47(0.14- 1.30)	0.23	4(11.1%)	9(37.5%)
Shigellae	2.5%	0.0%	NA	NA	3(8.3%)	0(0%)

* A single isolation was typical EPEC (*bfp+* gene)

Table 4a

Antibiotic resistance among the different diarrheagenic <i>E.coli</i> pathotypes in isolates from diarrhea (n=25) and control (n=22) in health center. No statistically significant differences were detected between cases and controls for any of the antibiotics and pathotypes tested, by Fisher exact test								
ANTIBIOTICS	DAEC, n(%)		EPEC*, n(%)		ETEC, n(%)		EAEC, n(%)	
	CASE(n=15)	CONTROL(n=7)	CASE(n=4)	CONTROL(n=7)	CASE(n=1)	CONTROL(n=4)	CASE(n=1)	CONTROL(n=4)
AM	13(86.7)	6(85.7)	2(50)	1(14.3)	1(25)	1(25)	1(100)	2(50)
AmC	0(0)	0(0)	1(25)	0(0)	0(0)	0(0)	0(0)	0(0)
CTX	1(6.7)	1(14.3)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
CF	4(26.7)	2(28.6)	2(50)	2(28.6)	1(100)	2(50)	0(0)	1(25)
C	0(0)	0(0)	0(0)	1(17.3)	0(0)	0(0)	0(0)	1(25)
CIP	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
SXT	15(100)	7(100)	1(25)	1(14.3)	1(100)	1(25)	1(100)	3(75)
CN	4(26.7)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
S	1(6.7)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Te	9(60)	4(57.1)	3(75)	3(42.9)	0(0)	0(0)	0(0)	3(75)
IPM	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
G	14(93.3)	6(85.7)	2(50)	2(28.6)	1(100)	1(25)	1(100)	3(75)
Any	15(100)	6(85.7)	3(75)	3(42.9)	1(100)	2(50)	1(100)	3(75)
antibiotic								

*Isolates of Typical EPEC and atypical EPEC

**Data on EIEC and Shigellae are not presented; due to small number of samples

Table 4b

Antibiotic resistance among the different diarrheagenic *E.coli* pathotypes in isolates from diarrhea (n=36) and control (n=24) in health center+hospital. No statistically significant differences were detected between cases and controls for any of the antibiotics and pathotypes tested, by Fisher exact test

ANTIBIOTICS	DAEC, n(%)		EPEC*, n(%)		ETEC, n(%)		EAEC, n(%)	
	CASE(n=18)	CONTROL(n=7)	CASE(n=4)	CONTROL(n=9)	CASE(n=6)	CONTROL(n=4)	CASE(n=1)	CONTROL(n=4)
AM	14(77.8)	9(85.7)	2(50)	2(22.2)	4(66.7)	1(25)	1(100)	2(50)
AmC	0(0)	0(0)	1(25)	0(0)	1(16.7)	0(0)	0(0)	0(0)
CTX	1(5.6)	1(14.3)	0(0)	0(0)	1(16.7)	0(0)	0(0)	0(0)
CF	5(27.8)	2(28.6)	2(50)	2(22.2)	4(66.7)	2(50)	0(0)	1(25)
C	0(0)	0(0)	0(0)	1(11.1)	0(0)	0(0)	0(0)	1(25)
CIP	0(0)	0(0)	0(0)	0(0)	1(16.7)	0(0)	0(0)	0(0)
SXT	17(94.4)	6(85.7)	1(25)	2(22.2)	4(66.7)	1(25)	1(100)	3(75)
CN	4(22.2)	0(0)	0(0)	0(0)	1(16.7)	0(0)	0(0)	0(0)
S	2(11.1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Te	11(61.1)	4(57.1)	3(75)	4(44.4)	1(16.7)	0(0)	0(0)	3(75)
IPM	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
G	15(83.3)	6(85.7)	2(50)	3(33.3)	4(66.7)	1(25)	1(100)	3(75)
Any	17(94.4)	6(85.7)	3(75)	4(44.4)	4(66.7)	2(50)	1(100)	4(100)
antibiotic								

*Isolates of Typical EPEC and atypical EPEC

**Data on EIEC and Shigellae are not presented; due to small number of samples

Table 5a

Antibiotic resistance among all diarrheagenic <i>E.coli</i> pathotypes in isolates from diarrhea case (n=25) and control (n=22) samples from health center					
ANTIBIOTICS	Case n(%)	Control n(%)	p-value*	OR(95%CI)	p-value
Ampicillin	19(76)	10(45.5)	0.03	3.8(1.09-13.17)	0.04
Amoxicillin-clavulanic acid	1(4)	0(0)	NA	NA	NA
Cefotaxime	1(4)	1(4.6)	0.93	0.88(0.05-14.87)	0.93
Cephalothin	8(32)	7(31.8)	0.99	1.0(0.29-3.45)	0.99
Chloramphenicol	0(0)	2(9.1)	NA	NA	NA
Ciprofloxacin	0(0)	0(0)	NA	NA	NA
Sulfamethoxazole-trimethoprim	21(84)	11(50)	0.01	5.3(1.35-20.4)	0.02
Gentamicin	4(16)	0(0)	NA	NA	NA
Streptomycin	1(4)	0(0)	NA	NA	NA
Tetracycline	13(52)	10(45.5)	0.65	1.3(0.41-4.10)	0.65
Imipenem	0(0)	0(0)	NA	NA	NA
Sulfisoxazole	22(88)	12(54.6)	0.01	6.1(1.41-26.56)	0.02
Any antibiotic	24(96)	14(63.6)	0.0032	13.7(1.55-121.42)	0.02
Multidrug resistance	22(88)	12(54.6)	0.01	6.11(1.23-30.30)	0.01

*Chi square test was used to the comparison between cases and controls (p≤0.05)

Table 5b

Antibiotic resistance among all diarrheagenic <i>E.coli</i> pathotypes in isolates from diarrhea case (n=36) and control (n=24) samples from health center+hospital					
ANTIBIOTICS	Case n(%)	Control n(%)	p-value*	OR(95%CI)	p-value
Ampicillin	26(72.2)	11(45.8)	0.04	3.07(1.04-9.09)	0.04
Amoxicillin-clavulanic acid	3(8.3)	0(0)	NA	NA	NA
Cefotaxime	2(5.6)	1(4.2)	0.81	1.35(0.12-15.81)	0.81
Cephalothin	13(36.11)	7(29.2)	0.57	1.37(0.45-4.17)	0.58
Chloramphenicol	3(8.3)	2(8.3)	1.0	1.0(0.15-6.48)	1.0
Ciprofloxacin	1(2.8)	0(0)	NA	NA	NA
Sulfamethoxazole-trimethoprim	29(80.56)	12(50)	0.01	4.14(1.31-13.08)	0.02
Gentamicin	5(13.9)	0(0)	NA	NA	NA
Streptomycin	2(6)	0(0)	NA	NA	NA
Tetracycline	19(52.8)	11(45.8)	0.60	1.32(0.47-3.72)	0.60
Imipenem	0(0)	0(0)	NA	NA	NA
Sulfisoxazole	29(80.6)	13(54.2)	0.03	3.5(1.10-11.09)	0.03
Any antibiotic	32(88.9)	15(62.5)	0.02	4.8(1.27-18.11)	0.02
Multidrug resistance	29(80.6)	13(54.2)	0.02	3.5(1.10-11.08)	0.03

*Chi square test was used to the comparison between cases and controls (p≤0.05)

PART IV

ANNEXES

Table 6

Risk factors associated with the presence of pathogens in the health center +hospital		
Factors	OR, CI 95%	<i>p</i> -value
Age	1.04(1.00-1.08)	0.02
Sex	2.47(0.86-7.14)	0.09
Medical use	1.6(0.54-4.68)	0.39
Trip in the last year	0.7(0.23-2.05)	0.52
Trip in the last week	1(0.25-3.99)	1.00
Use flush toilet	1.32(0.36-4.92)	0.68
Use diaper	1.77(0.32-9.99)	0.52
Use latrine	0.2(0.02-2.05)	0.18
Use of water purchased	3.14(0.61-16.31)	0.17
Internal water supply at home	1.07(0.34-3.36)	0.91
Water purchased and internal water	0.14(0.01-1.37)	0.09
Water treatment before consumption	0.24(0.07-0.77)	0.02
Boil the water	0.24(0.07-0.77)	0.02
Contact with animals	0.54(0.19-1.54)	0.25